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March 30, 2009

David Saliwanchik

David R. Saliwanchik, Patent Attorney

REQUEST FOR CERTIFICATE OF CORRECTION UNDER 37 CFR 1.322
Docket No. BB.138

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Bernd Bufe *et al.*
Issued	:	August 19, 2008
Patent No.	:	7,413,867
For	:	Method for the Identification of Antagonists of a Phenylthiocarbamide/Bitter Taste Receptor
Conf. No.	:	7026

Mail Stop Certificate of Corrections Branch
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

REQUEST FOR CERTIFICATE OF CORRECTION
UNDER 37 CFR 1.322 (OFFICE MISTAKE)

Sir:

A Certificate of Correction for the above-identified patent has been prepared and is attached hereto.

In the left-hand column below is the column and line number where errors occurred in the patent. In the right-hand column is the page and line number in the application where the correct information appears.

Patent Reads:	**Substitute Specification dated January 19, 2008 Reads:**
Column 6, Line 41 "Cobillger"	Page 9, Lines 6 and 7 --Cobinger--
Column 7, Line 7 "snRNA"	Page 9, Line 31 --scRNA--
Column 9, Line 34 "six, live, four"	Page 13, Line 15 --six, five, four--
Column 19, Line 12 "2-nitrophenylβ-D-glucoside"	Page 24, Lines 19 and 20 --2-nitrophenyl-β-D-glucoside--
Column 21, Line 33 "N,N-dimethylthioforminamide"	Page 28, Line 21 --N,N-dimethylthioformamide--
Column 22, Line 1 "with —O—R^1, in which R_1"	Page 29, Line 10 --with -O-R_1 in which R_1"
Column 24, Line 57 "lP3"	Page 33, Line 28 --IP_3--
Column 25, Line 57 "which arc required"	Page 35, Line 11 --which are required--
Column 28, Line 23 "IISV"	Page 39, Line 1 --HSV--
Column 29, Line 52 "$(EC_{50}/x)_{nH}$),"	Page 40, Line 33 --$(EC_{50}/x)^{nH}$),--
Column 30, Lines 65 and 66 "phenyl-alpha-Dglti-copyranoside"	Page 42, Lines 21 and 22 --phenyl-alpha-D-glucopyranoside--
Column 31, Lines 65 and 66 "arrestings"	Page 44, Line 15 --arrestins--
Column 32, Line 26 "gustdicin"	Page 44, Line 10 --gustducin--

Column 32, Line 38	Page 45, Line 9
"phosplolipase"	--phospholipase--

A true and correct copy of pages 9, 13, 24, 28, 29, 33, 35, 39, 40, 42, 44 and 45 of the Substitute Specification dated January 19, 2008, which supports Applicants' assertion of the errors on the part of the Patent Office accompanies this Certificate of Correction.

Approval of the Certificate of Correction is respectfully requested.

Respectfully submitted,

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DRS/jil

Attachments: Pages 9, 13, 24, 28, 29, 33, 35, 39, 40, 42, 44 and 45 of the Substitute Specification dated January 19, 2008
Certificate of Correction

1:237-251), herpes viruses, in particular Herpes simplex virus (HSV-1, Carlezon, W.A. et al. (2000) Crit. Rev. Neurobiol.), baculovirus, retrovirus, adeno-associated-virus (AAV, Carter, P.J. and Samulski, R.J. (2000) J. Mol. Med. 6:17-27), rhinovirus, human immune deficiency virus (HIV), filovirus and engineered versions thereof (see, for example, Cobinger G. P. et al (2001) Nat. Biotechnol. 19:225-30), virosomes, "naked" DNA liposomes, and nucleic acid coated particles, in particular gold spheres. Particularly preferred are viral vectors like adenoviral vectors or retroviral vectors (Lindemann et al. (1997) Mol. Med. 3:466-76 and Springer et al. (1998) Mol. Cell. 2:549-58). Liposomes are usually small unilamellar or multilamellar vesicles made of cationic, neutral and/or anionic lipids, for example, by ultrasound treatment of liposomal suspensions. The DNA can, for example, be ionically bound to the surface of the liposomes or internally enclosed in the liposome. Suitable lipid mixtures are known in the art and comprise, for example, DOTMA (1, 2-Dioleyloxpropyl-3-trimethylammoniumbromid) and DPOE (Dioleoylphosphatidylethanolamin) which both have been used on a variety of cell lines.

Nucleic acid coated particles are another means for the introduction of nucleic acids into cells using so called "gene guns", which allow the mechanical introduction of particles into the cells. Preferably the particles itself are inert, and therefore, are in a preferred embodiment made out of gold spheres.

In a further aspect the polynucleotide of the present invention is operatively linked to expression control sequences allowing expression in prokaryotic and/or eukaryotic host cells. The transcriptional/translational regulatory elements referred to above include but are not limited to inducible and non-inducible, constitutive, cell cycle regulated, metabolically regulated promoters, enhancers, operators, silencers, repressors and other elements that are known to those skilled in the art and that drive or otherwise regulate gene expression. Such regulatory elements include but are not limited to regulatory elements directing constitutive expression like, for example, promoters transcribed by RNA polymerase III like , e.g., promoters for the snRNA U6 or scRNA 7SK gene, the cytomegalovirus hCMV immediate early gene, the early or late promoters of SV40 adenovirus, viral promoter and activator sequences derived from, e.g. , NBV, HCV, HSV, HPV, EBV, HTLV, MMTV or HIV; which allow inducible expression like, for example, CUP-1 promoter, the tet-repressor as employed, for example, in the tet-on or tet-off systems, the lac system, the trp system; regulatory elements directing tissue specific expression, preferably taste bud specific ex-

40%; 50%; 60%; 70%; 80%; 90%; 95%; 98%; 99%; 99.5%; or 100% or even more) of the ability of the full-length TAS2R to bind to a bitter substance to which the full-length TAS2R binds. Binding assays and bitter substances are described herein. Further bitter substances can be identified by the binding assays and bitter taste receptor activity assays described herein. The polypeptides embraced by the invention also include fusion proteins that contain either a full-length TAS2R polypeptide or a functional fragment of it fused to an unrelated amino acid sequence. The unrelated sequences can be additional functional domains or signal peptides. Signal peptides are described in greater detail and exemplified below.

The polypeptides can be any of those described above but with not more than 50 (e.g., not more than: 50, 45, 40, 35, 30, 25, 20, 15, 14, 13, 12, 11, 10, nine, eight, seven, six, five, four, three, two, or one) conservative substitutions. Conservative substitutions typically include substitutions within the following groups: glycine and alanine; valine, isoleucine, and leucine; aspartic acid and glutamic acid; asparagine, glutamine, serine and threonine; lysine, histidine and arginine; and phenylalanine and tyrosine. All that is required of a polypeptide having one or more conservative substitutions is that it has at least 20% (e.g., at least: 20%; 30%; 40%; 50%; 60%; 70%; 80%; 90%; 95%; 98%; 99%; 99.5%; or 100% or even more) of the ability of the wild-type, full-length TAS2R to bind to a bitter substance, preferably the ability to release intracellular calcium, when expressed in a cellular system.

The polypeptides can be purified from natural sources (e.g., blood, serum, plasma, tissues or cells such as normal tongue cells or any cell that naturally produces the relevant TAS2R polypeptides). Smaller peptides (less than 50 amino acids long) can also be conveniently synthesized by standard chemical means. In addition, both polypeptides and peptides can be produced by standard *in vitro* recombinant DNA techniques and *in vivo* transgenesis, using nucleotide sequences encoding the appropriate polypeptides or peptides. Methods well-known to those skilled in the art can be used to construct expression vectors containing relevant coding sequences and appropriate transcriptional/translational control signals. See, for example, the techniques described in Sambrook et al., *Molecular Cloning: A Laboratory Manual* (2nd Ed.) [Cold Spring Harbor Laboratory, N.Y., 1989], and Ausubel et al., *Current Protocols in Molecular Biology* [Green Publishing Associates and Wiley Interscience, N.Y., 1989].

embodiment the polypeptides and agonist employed together in above process are selected from the group consisting of:

(a) the polypeptide encoded by the polynucleotide outlined above as determined by SEQ ID NO: 1 and SEQ ID NO: 2 and the agonist selected from the group consisting of acetylthiourea, N,N-dimethylthioformamide, N,N'-diphenylthiourea, N-ethylthiourea, 2-imidazolidinethione, 4(6)-methyl-2-thiouracil, N-methylthiourea, phenylthiocarbamid, 6-phenyl-2-thiouracil, 6-propyl-2-thiouracil, tetramethylthiourea, thioacetamide, thioacetanilide, 2-thiobarbituric acid, and 2-thiouracil and functional derivatives thereof;

(b) the polypeptide encoded by the polynucleotide of claim 1 or 2 as determined by SEQ ID NO: 9 and SEQ ID NO: 10 and the agonist selected from the group consisting of saccharin and functional derivatives thereof;

(c) the polypeptide encoded by the polynucleotide of claim 1 or 2 as determined by SEQ ID NO: 11 and SEQ ID NO: 12 and the agonist selected from the group consisting of saccharin and acesulfame K and functional derivatives thereof;

(d) the polypeptide encoded by the polynucleotide of claim 1 or 2 as determined by SEQ ID NO: 13 and SEQ ID NO: 14 and the agonist selected from the group consisting of absinthine and functional derivatives thereof;

(e) the polypeptide encoded by the polynucleotide of claim 1 or 2 as determined by SEQ ID NO: 15 and SEQ ID NO: 16 and the agonist selected from the group consisting of absinthine and functional derivatives thereof;

(f) the polypeptide encoded by the polynucleotide of claim 1 or 2 as determined by SEQ ID NO: 19 and SEQ ID NO: 20 and the agonist selected from the group consisting of absinthine and functional derivatives thereof;

(g) the polypeptide encoded by the polynucleotide of claim 1 or 2 as determined by SEQ ID NO: 37 and SEQ ID NO: 38 and the agonist selected from the group consisting of strychnine, brucine, denatonium benzoate, and absinthine and functional derivatives thereof;

(h) the polypeptide encoded by the polynucleotide of claim 1 or 2 as determined by SEQ ID NO: 41 and SEQ ID NO: 42 and the agonist selected from the group consisting of tyrosine, preferably L-tyrosine, and other bitter tasting amino acids including, e.g., leucine, histidine phenylalanine and tryptophan, and functional derivatives thereof; and

(i) the polypeptide encoded by the polynucleotide of claim 1 or 2 as determined by SEQ

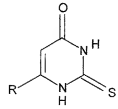
through an allosteric mechanism, and/or stabilize the receptor in the inactive conformation, and/or bind reversibly or irreversibly, and/or weaken receptor G protein interaction, and/or interfere with G protein activation).

Similarly, in another embodiment of the invention, it has been found that the so-called hTAS2R10 receptor is activated by strychnine, and strychnine analogues such as brucine as well as by denatonium benzoate, absinthine and other alkaloids with (a) ring system(s). Strychnine and its analogues are also useful phytochemicals that find use in medicines and homeopathic treatments.

In another embodiment of the invention, it has been found that the so-called hTAS2R14 receptor is activated by tyrosine, in particular L-tyrosine, and other bitter tasting amino acids including leucine, histidine, phenylalanine and tryptophan.

In another embodiment of the invention, it has been found that the so-called hTAS2R38 receptor is activated by acetylthiourea, N,N-dimethylthioformamide, N,N'-diphenylthiourea, N-ethylthiourea, 2-imidazolidinethione, 4(6)-methyl-2-thiouracil, N-methylthiourea, phenylthio-carbamid, 6-phenyl-2-thiouracil, 6-propyl-2-thiouracil, tetramethylthiourea, thioacetamide, thioacetanilide, 2-thiobarbituric acid, and 2-thiouracil.

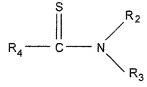
From these studies certain inferences can be drawn regarding the affinity of the compounds, which activate the hTAS2R38 receptor. Thus, for the promotion of activation derivatives of 2-thiouracil according to following formula are preferred compounds.



Whereas R in this formula can be hydrogen, it is preferred that R is a substituent selected from C_1-C_{10} alkyl, which may be branched, linear or cyclic as appropriate, particularly preferred alkyls are methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, tert-butyl, n-pentyl or iso-pentyl residues; lower alkenyl residues, preferably having two, three, four or five carbon atoms; lower alkynyl residues, preferably having two, three, four or five carbon atoms,

which can in a preferred embodiment be further substituted with F, Cl, Br, NH_2, NO_2, OH, SH, NH, CN, aryl, heteroaryl, COH or COOH group; heteroaryl, e.g. benzofuran and cumarin; aryl, e.g. phenyl, naphtyl; F, Cl, Br, NH_2, NO_2, OH, SH, NH, CN, aryl, alkylaryl, heteroaryl, alkylheteroaryl, COH or COOH group. In a further embodiment the carbon atom at the 4 position can substituted with -O-R_1 in which R_1 can have the same meaning as outlined above for R.

Another general structure of compounds having affinity for hTAS2R38 and which are thus suitable for activation of hTAS2R38 is depicted by the following formula:



In this formula R_2, R_3, and R_4 can each independently of each other have the meaning H; alkyl, in particular lower alkyl (C_1-C_5, e.g. methyl, ethyl, n-propyl, isopropyl, n-butyl, iso-butyl, tert-butyl, n-pentyl or iso-pentyl); substituted alkyl; alkenyl, in particular lower alkenyl (C_2-C_5); substituted alkenyl; alkinyl, in particular lower alkinyl (C_2-C_5); substituted alkinyl: alkanal, in particular lower alkanal (e.g. -$COCH_3$, -$COCH_2CH_3$, -$COCH_2CH_2CH_3$); aryl, in particular phenyl; substituted aryl; heteroaryl; substituted heteroaryl; alkylaryl, in particular benzyl; substituted alkylaryl; alkylheteroaryl; substituted alkylheteroaryl aminoalkyl, in particular –$NHCH_3$, –$NHCH_2CH_3$, –$N(CH_3)_2$; substituted aminoalkyl; aminoketone, in particular -$NHCOCH_3$; substituted aminoketone; aminoaryl, in particular –NH-Ph; substituted aminoaryl; CN; NH_2; Halogen, in particular F, Cl, and Br; NO_2. In a preferred embodiment R_2 or R_3 and R_4 can form a ring, preferably a four, five, six, seven or eight membered hetero cycle, which in a preferred embodiment is an aromatic hetero cycle. The residue of R_2 or R_3, which is not involved in the formation of the ring structure can have any of the meanings as outlined above. In a further preferred embodiment at least one of R_2 or R_3 has the meaning alkanal, preferably lower alkanal as outlined above. In case that only one of R_2 or R_3 has the meaning alkanal, than the other substituent preferably has the meaning H.

recovered. Alternatively, the recombinant protein may be recovered from the culture medium in which the recombinant cells had been cultured.

The activity of any of the receptors described herein can be assessed using a variety of *in vitro* and *in vivo* assays to determine functional, chemical, and physical effects, e.g., measuring ligand binding, secondary messengers (e.g., cAMP, cGMP, IP_3, DAG, or Ca^{2+}) ion flux, phosphorylation levels, transcription levels, neurotransmitter levels, and the like. Furthermore, such assays can be used to test for inhibitors of the receptors as is well known in the art.

Samples or assays that are treated with a potential receptor inhibitor may be compared to control samples without the test compound, to examine the extent of modulation. Control samples (untreated with inhibitors) are assigned a relative receptor activity value of 100. Inhibition of receptor activity is achieved when the receptor activity value relative to the control is lower, and conversely receptor activity is enhanced when activity relative to the control is higher.

The effects of the test compounds upon the function of the receptors can be measured by examining any of the parameters described above. Any suitable physiological change that affects receptor activity can be used to assess the influence of a test compound on the receptors of this invention. When the functional consequences are determined using intact cells or animals, one can measure a variety of effects such as changes in intracellular secondary messengers such as Ca^{2+}, IP_3 or cAMP.

Preferred assays for G-protein coupled receptors include cells that are loaded with ion sensitive dyes to report receptor activity. In assays for identifying modulatory compounds, changes in the level of ions in the cytoplasm or membrane voltage will be monitored using an ion sensitive or membrane voltage fluorescent indicator, respectively. For G-protein coupled receptors, promiscuous G-proteins such as G.alpha.15 and G.alpha.16 and chimeric G-proteins can be used in the assay of choice (see, for example, Wilkie et al., *Proc. Nat. Acad. Sci. USA* **88**, 10049-10053 (1991)). Such promiscuous G-proteins allow coupling of a wide range of receptors.

(2) determining whether the potential agonist or antagonists agonizes or antagonizes the activity of the signalling molecule.

The activity of the signalling molecule and the increase or decrease of that activity in response to the potential agonist or antagonist can be determined as outlined above with respect to the identification of bitter receptor taste activity. The respectively indicated percent increases or decreases of the activity, which are required to qualify as antagonist or agonist do apply mutatis mutandis. Additionally the term "contacting" has the meaning as outlined above. Preferably the signalling molecule and/or the promiscuous G-protein has been introduced into the cell. The type of cell, which are preferred are those indicated above.

In yet another embodiment, the ligand-binding domains of the receptors can be employed *in vitro* in soluble or solid-state reactions to assay for ligand binding. Ligand binding in a receptor, or a domain of a receptor, can be tested in solution, in a bilayer membrane attached to a solid phase in a lipid monolayer or vesicles. Thereby, the binding of a modulator to the receptor, or domain, can be observed using changes in spectroscopic characteristics, e.g. fluorescence, absorbance or refractive index; or hydrodynamic (e.g. shape), chromatographic, or solubility properties, as is generally known in the art.

The compounds tested as modulators of the receptors can be any small chemical compound, or a biological entity, such as a protein, sugar, nucleic acid or lipid. Typically, test compounds will be small chemical molecules. Essentially any chemical compound can be used as a potential modulator or ligand in the assays of the invention, although knowledge of the ligand specificity of an individual receptor would enable the skilled person to make an intelligent selection of interesting compounds. The assays may be designed to screen large chemical libraries by automating the assay steps and providing compounds from any convenient source to assays, which are typically run in parallel (e.g., in microtiter formats on microtiter plates in robotic assays). The skilled person will understand that there are many suppliers of libraries of chemical compounds.

Assays may be run in high throughput screening methods that involve providing a combinatorial chemical or peptide library containing a large number of potential therapeutic, or tastant compounds (that are potential ligand compounds). Such libraries are then screened

We compared the amino acid sequences using the AlignX program of the Vector NTI™ Suite (InforMax).

Using the above-described method, DNA sequences encoding all 24 bitter receptors identified by applicant were cloned. As indicated above, they were derived by a PCR-based method using genomic DNA as the template. Since all of the 24 genomic sequences lack introns, the DNA clones obtained had the same sequences as corresponding cDNA clones derived by reverse transcription-PCR (RT-PCR) of mRNA from cells expressing the relevant polypeptides would have.

EXAMPLE 2: Immunocytochemistry

Batches of HEK293 cells were separately transiently transfected with expression vectors (pCDN5/FRT; Invitrogen) containing each of the 24 above described coding sequences using lipofectamine 2000 (Invitrogen) and aliquots of the resulting cell populations were separately seeded on polylysine-coated coverslips. At 24 h post transfection they were washed with phosphate buffered saline (PBS), cooled on ice and added 20 microgram / ml biotin-labeled concanavalin A (Sigma) for 1 h, which binds to cell surface glycoproteins. Thereafter, the cells were fixed for 5 min in methanol/acetone (1:1) and then permeabilized for 4 min with 0.25% Triton X-100. In order to reduce nonspecific binding the coverslips were incubated in 2% goat serum. Thereafter, anti-HSV glycoprotein D antiserum (Novagen, 1:10,000) was added to detect the chimeric receptors that, as described above, would have a HSV glycoprotein epitope fused to their C-temini, and Texas Red-Avidin D (Vector, 1:200) has added to stain the cell surface and incubation continued overnight at 4 °C. Such C-termini are intracellular and for this reason it is necessary to permeabilize the cells to permit entry of the HSV glycoprotein D epitope-specific antibody molecules into them. After washing (5x in PBS, RT) Alexa488-conjugated goat anti-mouse antiserum (Molecular Probes, 1:1000) was added and incubation continued at room temperature for 1 h. Finally, the cells were embedded in Fluorescent Mounting Medium (Dako) and analyzed using a Leica TCS SP2 Laser Scan Inverted microscope. The preparations were scanned sequentially with an argon/krypton laser (488 nm) to excite the Alexa488 dye and with a green-helium-neon laser (543 nm) to excite the Texas Red dye. The spectral detector recorded light emission at 510-560 nm and 580-660 nm, respectively. Images of 1024 X 1024 pixels were processed with Corel PHOTO-PAINT 10.0 (Corel Corporation) and

printed on a Tektronix color laser printer. The immunocytochemical data permitted calculation of the proportion of cells expressing recombinant receptors (green fluorescent cells divided by total cell number in a microscopic field) and the proportion of cells that display expression of TAS2Rs at the plasma membrane level (number of cells with colocalization of green and red fluorescence divided by the number of green fluorescent cells). Of the 24 transfectant lines tested, all were found to express the encoded polypeptides. The proportion of receptor-expressing cells in the various transfectant lines ranged from about 10% to about 35%.

EXAMPLE 3: Heterologous Expression of hTAS2R Receptors

A fluorescence imaging plate reader (FLIPR, Molecular Devices) was used to functionally screen cell populations transiently transfected with expression vectors encoding the above-described 24 bitter receptors and to establish concentration-response curves for hTAS2R16 and hTASR10. The single-cell calcium imaging technique was also employed to demonstrate receptor selectivity and crossdesensitization. For the FLIPR experiments the HEK293/15 cells were grown to 50% confluence. The cells were then seeded at a density of 3×10^3 cells per well into 96-well black-wall, clear-bottom microtiter plates (Greiner). After 48 h the cells in each well were transfected using Lipofectamine 2000 and 24-30 h later were loaded with FIuo4AM (Molecular Probes). Thereafter they were stimulated with bitter compounds (SigmaAldrich, further purified by reversed-phase HPLC to > 99% purity). Calcium signals were recorded simultaneously from each well at 1 Hz at 510 nm after excitation at 488 nm and the recordings were corrected for cell density. The responses of five wells containing cells expressing the same receptor and that received the same stimulus (i.e., the same compound at the same concentration) were averaged. Calcium traces were subtracted that were determined in triplicate of mock-transfected cells stimulated with the same concentration of tastant. The calculations rest on at least four independent transfection experiments. Plots of the amplitudes versus concentrations fitted by nonlinear regression to the function $f(x)=100/(1+(EC_{50}/x)^{nH})$, with x = agonist concentration and nH = Hill coefficient permitted calculation of EC_{50} values and threshold values of activation.

EC_{50} and threshold values obtained with hTAS2R16-expressing transfectants are shown in Table 1 below and the results are described in Example 4.

In separate experiments, hTAS2R10-expressing transfectants were found to have a thresh-

EXAMPLE 4: Human Taste Experiments

15 experienced panelists in a sensory panel room at 22-25 °C determined bitter thresholds on three different sessions using a triangle test with tap water as solvent, according to methodology set out in *J. Agric. Food Chem.*, **49**, 231-238 (2001), or Mailgaard M et al, "*Sensory Evaluation Techniques*" (CRC Press LLC, New York 1999). For dose-response relations, bitter tastant concentration series were presented to 10 trained panelists in random order. The panelists ranked the samples in increasing order of intensity and, for each concentration, evaluated bitterness intensity on a scale from 0 to 5 (ref. 24). The dose-response curves of three different sessions were averaged. The intensity values between individuals and separate sessions differed by not more than 0.5 units.

To investigate adaptation, the 8 panelists first maintained aqueous solutions (5 ml) of phenyl-β-D-glucopyranoside (8 mM), phenyl-alpha-D-glucopyranoside (180 mM), salicin (8 mM), or helicin (8 mM) for 15 s in their oral cavities and evaluated the bitter intensity as described above. After 30 min, they kept a denatonium benzoate solution (5 ml, 0.0003 mM) for 15 s in their mouth and evaluated its bitterness. The panelists spat off the denatonium benzoate solution, took up the phenyl-β-D-glucopyranoside or the phenyl-alpha-D-glucopyranoside solutions orally for 120 s or 180 s and judged their bitterness intensity after 15, 30, 60, 120 and 180 s. Thereafter, the panelists spat off these solutions and then sequentially took up salicin, helicin (5 ml, 8 mM) and denatonium benzoate (5 ml, 0.0003 mM) and evaluated bitterness intensities of these solutions after 15 s. After an additional 30 min, the first experiment was repeated. The data of three different sessions for each panelist were averaged. Intensity values between individuals and separate sessions differed by not more than ±0.5 units.

Results of in vitro assays (FLIPR) and human taste experiments are shown in Table 1 below.

ceptor for these class of bitter compounds. In contrast, the related structures (see compounds 10 and 11) show 90- to 400-fold higher Threshold Concentrations, which indicates that this receptor is rather selective, and that these bitter compounds activate different receptors.

Adaptation frequently occurs in sensory systems and means that stimuli elicit reduced responses upon prolonged or repeated stimulus presentations. Repeated stimulation of hTAS2R16-expressing cells with phenyl-beta-D-glucopyranoside resulted in largely diminished responses to salicin as well. This cross-desensitization occurred among the other tested beta-pyranosides and was fully reversible. It resembles homologous desensitization of agonist-occupied heptahelical receptors mediated by GRKs, i.e. specific kinases, and arrestins. We also observed adaptation in the human test panel that initially scored phenyl-beta-D-glucopyranoside, salicin and helicin as equally intensely bitter. The bitterness of phenyl-beta-D glucopyranoside declined during prolonged stimulation and the test panel perceived salicin and helicin also as less bitter, but not the unrelated bitter substance denatonium benzoate, which cannot activate TAS2R16. Adaptation was fully reversible. On the opposite, the phenyl-alpha-D-glucopyranoside failed to cross-adapt with all tested beta-D-glucopyranosides, although its own bitter response desensitized strongly. This indicates that beta-glucopyranosides signal through a common mechanism most likely involving hTAS2R16 as a bitter taste receptor while the alpha-isomer activates a separate receptor. A recent human psychophysical study also revealed cross-adaptation amongst two bitter amino acids but not between the two bitter amino acids and urea, suggesting the existence of distinct receptors for the bitter amino acids and urea. Although most, if not all, bitter receptors are present in the same subset of taste receptor cells, adaptation to specific bitter stimuli can be explained if bitter receptors were subject to homologous desensitization.

EXAMPLE 5: Heterologous Expression of hTAS2R

Transient transfection of TAS2Rs into HEK-293T-Gα16gustducin44 cells. We cloned the DNAs of all human putative bitter responsive receptors into pCDNA5/FRT (Invitrogen) by PCR-methods and transiently transfected the plasmids with lipofectamine 2000 (Invitrogen) into HEK-293T-Gα16gustducin44 cells grown to 50% confluence. These cells stably express a chimeric G protein constructed from human Gα16 and rat gustducin. Finally, we

seeded the transfected cells at a density of 3 x 10^3 cells per well into 96-well black-wall, clear-bottom microtiter plates (Greiner).

Co-transfection of TAS2Rs with gustducin and phospholipase-Cβ2 into HEK-293 cells. Alternatively, we transfected simultaneously plasmid DNAs encoding one of the TAS2Rs, phospholipase-Cβ2 and α-gustducin into HEK-293 cells using the lipofectamine method. Additional cotransfection of G-protein β and γ-subunits may improve the bitter tastant-induced responses. Thereafter, the transfected cells were seeded at a density of 3 x 10^3 cells per well into 96-well black-wall, clear-bottom microtiter plates (Greiner).

Fluorometric Imaging Plate Reader (FLIPR) assay 24-30 h later, the cells were loaded with 4 μM FLUO-4/AM (Molecular Probes) and 0.04% Pluronic F-127 (Molecular Probes) in Hepes-buffered saline (HBS), 140 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 10 mM Hepes, 10 mM glucose and 2.5 mM probenicide, pH 7.4, for 1 hour at 37°C. Thereafter, cells were gently washed in HBS by an automated plate washer (Denley Cellwash, Labsystems) and transferred to the FLIPR (Molecular Devices). The FLIPR integrates an argon laser excitation source, a 96-well pipettor, and a detection system utilizing a Charged Coupled Device imaging camera. Fluorescence emissions from the 96 wells were monitored at an emission wavelength of 510 nm, after excitation with 488 nm (F488). Fluorescence data were collected 1 min before and 10 min after stimulation. Data were collected every 6 s before and every 1 s after agonist stimulation. 50 μl of 3x concentrated agonists were delivered within 2 s by the integrated 96-well pipettor to the wells containing 100 μl HBS. Agonist responses were quantified using the amplitudes of the fluorescence peaks. We averaged the responses of five wells containing cells expressing the same receptor and that received the same stimulus. Calcium traces were determined in triplicate of mock-transfected cells stimulated with the same concentration of tastant. EC_{50} values and plots of the amplitudes versus concentrations were derived from fitting the data by nonlinear regression to the function $f(x) = 100 / [1 + (EC_{50}/x)^{nH}]$, where x is the agonist concentration and nH is the Hill coefficient. The results for hTAS2R10 (Table II), hTAS2R14 (Table III), hTAS2R16 (Table IV), hTAS2R38 (Table V), hTAS2R43 (Table VI), hTAS2R44 (Table VII), hTAS2R45 (Table VIII), hTAS2R46 (Table IX) and hTAS2R (Table X) are shown below.

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 7,413,867

APPLICATION NO.: 10/528,630

DATED : August 19, 2008

INVENTORS : Bernd Bufe, Thomas Hofmann, Dietmar Krautwurst, Christina Kuhn, Wolfgang Meyerhof

It is certified that errors appear in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 6,
Line 41 "Cobillger" should read --Cobinger--.

Column 7,
Line 7 "snRNA" should read --scRNA--.

Column 9,
Line 34 "six, live, four" should read --six, five, four--.

Column 19,
Line 12 "2-nitrophenylβ-D-glucoside" should read --2-nitrophenyl-β-D-glucoside--.

Column 21,
Line 33 "N,N-dimethylthioforminamide" should read --N,N-dimethylthioformamide--.

Column 22,
Line 1 "with —O—R^1, in which R_1" should read --with -O-R_1 in which R_1--.

Column 24,
Line 57 "IP3" should read --IP_3--.

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UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 7,413,867

APPLICATION NO.: 10/528,630

DATED : August 19, 2008

INVENTORS : Bernd Bufe, Thomas Hofmann, Dietmar Krautwurst, Christina Kuhn, Wolfgang Meyerhof

It is certified that errors appear in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 25,
Line 57 "which arc required" should read --which are required--.

Column 28,
Line 23 "IISV" should read --HSV--.

Column 29,
Line 52 "$(EC_{50}/x)_{nH}$)," should read --$(EC_{50}/x)^{nH}$),--.

Column 30,
Lines 65 and 66 "phenyl-alpha-Dglti-copyranoside" should read --phenyl-alpha-D-glucopyranoside--.

Column 31,
Lines 65 and 66 "arrestings" should read --arrestins--.

Column 32,
Line 26 "gustdicin" should read --gustducin--.
Line 38 "phosplolipase" should read --phospholipase--.

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